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Abstract
Cell-free lysates of the strict anaerobe Oxalobacter formigenes contained the following enzymatic activities: oxalyl coenzyme A reductase, glyoxylate carboligase, tartronic semialdehyde reductase, and glycerate kinase. NAD(P)-linked formate dehydrogenase, serine-glyoxylate aminotransferase, and NAD(P) transhydrogenase activities were not detected. These results support the hypothesis that O. formigenes assimilates carbon from oxalate by using the glycerate pathway, whereby oxalate is reduced to 3-phosphoglycerate before entering common biosynthetic pathways.

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NOTES

Anabolic Incorporation of Oxalate by *Oxalobacter formigenes*

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Cell-free lysates of the strict anaerobe *Oxalobacter formigenes* contained the following enzymatic activities: oxalyl coenzyme A reductase, glyoxylate carboligase, tartronic semialdehyde reductase, and glycerate kinase. NAD(P)-linked formate dehydrogenase, serine-glyoxylate aminotransferase, and NAD(P) transhydrogenase activities were not detected. These results support the hypothesis that *O. formigenes* assimilates carbon from oxalate by using the glycerate pathway, whereby oxalate is reduced to 3-phosphoglycerate before entering common biosynthetic pathways.

Oxalobacter formigenes is a gram-negative anaerobic bacterium that inhabits the gastrointestinal tract of many warm-blooded animals (3, 10). Oxalate is the only substrate that supports the growth of this organism, although small amounts of acetate (0.5 mM) are also required. Aerobic bacteria that metabolize oxalate produce CO_2 and formate and then oxidize formate via formate dehydrogenase (2, 17). In contrast, *O. formigenes* (3) and other anaerobic oxalate-degrading bacteria (11, 26) produce formate as a major end product of oxalate catabolism. There is no information regarding the mechanisms used for the assimilation of carbon from oxalate by anaerobic bacteria. Aerobic oxalate-degrading bacteria use either of two pathways to convert oxalate to 3-phosphoglycerate, the glycerate pathway, and a variation of the serine pathway. The key enzymes that differentiate the two pathways are glyoxylate carboligase (glycerate pathway) and serine-glyoxylate aminotransferase (serine pathway). The first step of both the serine and glycerate pathways is the activation of oxalate to oxalyl coenzyme A (oxalyl-CoA). Previous studies with *O. formigenes* indicated that oxalyl-CoA is an intermediate in oxalate catabolism (3). In this study we assayed cell-free lysates of *O. formigenes* for enzymes in both the serine and glycerate pathways to determine whether oxalate was assimilated by either of these routes.

O. formigenes OxB (ATCC 35274) was grown under CO_2 in a 12-liter fermentor in medium B (3) modified such that the oxalate concentration was 100 mM. The pH of the medium was automatically maintained at 6.8 by the pH-dependent infusion of 0.8 M oxalic acid. Cell-free lysates were prepared by suspending cells in anaerobic phosphate buffer (100 mM; pH 6.7) containing 1 mM dithiothreitol and passing the bacteria through a French pressure cell. Cell membranes were pelleted by high-speed (62,000 × *g*) centrifugation, and the supernatant from this step was used for enzyme assays. In some instances, tests for activity in membrane fractions were also conducted. Protein was measured by a modification of the Lowry assay (19) using bovine serum albumin as the standard.

Assays for enzymatic activity were determined by previously described methods (Table 1) except as noted. Assay results were determined both aerobically and anaerobically (under an N_2 or argon atmosphere) to check for O_2 sensitivity. The data reported here are those collected from aerobic assays unless otherwise stated. Glyoxylate dehydrogenase was assayed under an argon atmosphere by the method of Quayle and Taylor (24). Tartronic semialdehyde was prepared enzymatically with glyoxylate carboligase purified from *Escherichia coli* (ATCC 8739) by the method of Gupta and Vennesland (15). The glyoxylate carboligase reaction was stopped with 200 μl of 50% trichloroacetic acid. Serine-glyoxylate aminotransferase was assayed by the procedure of Goodwin (14), except that the disappearance of serine and formation of glycine were measured by high-performance liquid chromatography (HPLC). Amino acids were detected as dabsylated derivatives by the procedure described by Allen (1) and modified by Anderson et al. (5). Glyoxylate carboligase was determined from measurements of CO_2 production in reactions conducted under an atmosphere of N_2 or argon (15). A gas chromatograph equipped with a thermal conductivity detector was used to measure CO_2 (8).

Cell extracts of *O. formigenes* contained oxalyl-CoA reductase, glyoxylate carboligase, tartronic semialdehyde reductase, glycerate kinase, and glyoxylate dehydrogenase activities (Table 1). These are the enzymes of the glycerate pathway (Fig. 1). The glycerate pathway is also utilized by "*Pseudomonas oxalaticus*" (22, 23), *Alcaligenes eutrophus* H16 (13), and *Thiobacillus novellus* (6) to convert oxalate to 3-phosphoglycerate. *O. formigenes* utilized NADH as a cofactor in the reduction of oxalyl-CoA to glyoxylate (oxalyl-CoA reductase) as does *A. eutrophus* (13). However, "*P. oxalaticus*" required NADPH for this reaction (21, 23). In "*P. oxalaticus*" extracts, this reaction is reversible (glyoxylate + NADP + CoA→oxalyl-CoA + NADPH). When the reverse reaction (glyoxylate dehydrogenase) was assayed in *O. formigenes* extracts, activity was detected with NADP but not with NAD. Hydroxypyruvate reductase activity was also detected, but its activity was about 1/10 of the activity observed for tartronic semialdehyde reductase. Serine-glyoxylate aminotransferase activity was not detected. Neither solu-

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TABLE 1. Activity of biosynthetic enzymes in cell-free lysates of *O. formigenes*

Enzyme	EC no.	Reference(s)	Assay measurement	Sp act (mean ± SD)[a]
Oxalyl-CoA reductase		20, 21	NADH oxidation	10 ± 1
		20, 21	NADPH oxidation	ND (<3)
Glyoxylate dehydrogenase	1.2.1.17	24	NAD reduction	ND (<5)
		24	NADP reduction	221 ± 35
Glyoxylate carboligase	4.1.1.47	15	CO_2 production	32 ± 7
Tartronic semialdehyde reductase	1.1.1.60	18	NADH oxidation	71 ± 6
		18	NADPH oxidation	51 ± 9
Glycerate kinase	2.7.1.31	16	NADH oxidation[b]	2 ± 0.6
Hydroxypyruvate reductase	1.1.1.29	16	NADH oxidation	6 ± 0.9
		16	NADPH oxidation	4 ± 0.6
Hydroxypyruvate reductase		9	Loss of hydroxypyruvate[c]	2 ± 0.6
Serine-glyoxylate aminotransferase	2.1.6.45	14	Loss of serine[c]	ND (<0.01)
NAD(P) transhydrogenase	1.6.1.1	25	(AcPy)NAD^{+}[d] reduction	ND (<2)

[a] Values given are means ± standard deviations of triplicate assays from two preparations of cell lysate. Units are nanomoles per minute per milligram of protein. ND, none detected (limit of detection given in parentheses).
[b] Assay coupled to lactate dehydrogenase measurement.
[c] Substrate loss measured by HPLC.
[d] (AcPy)NAD^{+}, 3-acetylpyridine adenine dinucleotide.

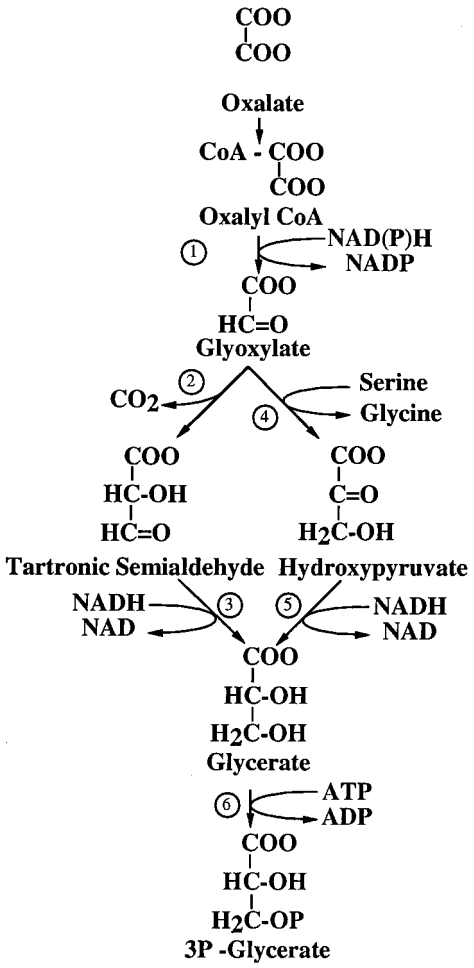

FIG. 1. The glycerate pathway (left branch) and the serine pathway (right branch). Enzymes of reactions: 1, oxalyl-CoA reductase; 2, glyoxylate carboligase; 3, tartronic semialdehyde reductase; 4, serine-glyoxylate aminotransferase; 5, hydroxypyruvate reductase; 6, glycerate kinase. 3P-Glycerate, 3-phosphoglycerate.

ble nor membrane bound NAD(P) transhydrogenase activities were detected.

Formate dehydrogenase (EC 1.2.1.2) was measured both by spectrophotometric methods (12) and by HPLC techniques (9). This activity was not detected when NAD or NADP was supplied as an electron acceptor in the spectrophotometric assay. However, when methyl or benzyl viologen dye was added (under N_2 or argon), cell extracts reduced the dye. The specific activity of the dye reduction reaction was calculated to be 1,900 nmol min^{-1} mg of $protein^{-1}$. Viologen dye was not reduced when formate was not included or with boiled lysate. Formyl-CoA, oxalate, acetate, carbon monoxide, formaldehyde, methanol, and glyoxylate did not serve as substrates in place of formate for viologen dye reduction. Preparations of cell membranes did not reduce viologen dye when formate was added. Formate dehydrogenase was also assayed by measuring the radiolabel in the CO_2 produced from [^{14}C]formate (17 mM; 6.15 kBq/ml). The latter reactions were stopped after 40 min by injecting 1 ml of 1 N NaOH through the rubber stoppers. [^{14}C]carbonate was released by acid trapped by diffusion into phenethylamine and counted with a scintillation counter (4). By this method, less than 1% of the ^{14}C was recovered as $^{14}CO_2$ during 40 min. The specific activity of formate dehydrogenase based on the amounts of $^{14}CO_2$ formed (4 nmol min^{-1} mg of $protein^{-1}$) was 500-fold less than the specific activity calculated by the viologen dye assay. Reaction mixtures that contained limiting concentrations of formate (on the basis of calculations from the viologen dye assay, 46.5 μM) were analyzed by HPLC, and no change in the concentration of formate was detected.

Both "*P. oxalaticus*" and *Methylobacterium extorquens* produce NAD-linked formate dehydrogenase (2, 17). In these aerobes the formation of NADH provides reducing equivalents for the cell and is also involved in ATP synthesis via an electron transport system. It seems unlikely that *O. formigenes* produces a fully functional formate dehydrogenase. Although an enzymatic reaction that reduced viologen dye in the presence of formate occurred, we were unable to directly measure the loss of formate by HPLC. We were also unable to measure appropriate amounts of $^{14}CO_2$ produced from [^{14}C]formate based on predictions from measurements with the viologen dye assay. Permeabilized-cell suspensions of other anaerobic oxalate-degrading bacteria, *Oxalobacter vibrioformis* and *Oxalophilus oxalicus*, also reduced viologen dye in the presence of

formate (11), even though formate accumulated as a major end product of oxalate metabolism.

The question of how *O. formigenes* obtains NADH to supply the reactions of the glyoxylate pathway remains. Ratios of CO_2 to formate produced from [^{14}C]oxalate indicate that growing cells oxidize more carbon from oxalate than do resting cells (7). It is possible that the NADP-linked glyoxylate dehydrogenase or the viologen dye-related formate dehydrogenase activities that were detected in cell-free lysates might somehow be involved in the generation of reducing power in *O. formigenes*. If the NADP-linked glyoxylate dehydrogenase activity observed is part of a branch pathway leading to the oxidation of both carbons of glyoxylate to CO_2, reducing equivalents needed for cell synthesis might be supplied. However, we have no evidence for this.

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